

## EXTENDED REPORT

# Tartrate resistant acid phosphatase (TRAP) positive cells in rheumatoid synovium may induce the destruction of articular cartilage

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**Objective:** To examine the role of tartrate resistant acid phosphatase (TRAP) positive mononuclear and multinucleated cells in the destruction of articular cartilage in patients with rheumatoid arthritis (RA).

**Methods:** The presence of TRAP positive cells in the synovial tissue of patients with RA was examined by enzyme histochemistry and immunohistochemistry. Expression of mRNAs for matrix metalloproteinases (MMPs) was assessed by the reverse transcriptase-polymerase chain reaction (RT-PCR) and northern blot analysis. Production of MMPs by mononuclear and multinucleated TRAP positive cells was examined by immunocytochemistry, enzyme linked immunosorbent assay (ELISA) of conditioned medium, and immunohistochemistry of human RA synovial tissue. In addition, a cartilage degradation assay was performed by incubation of <sup>35</sup>S prelabelled cartilage discs with TRAP positive cells.

**Results:** TRAP positive mononuclear cells and multinucleated cells were found in proliferating synovial tissue adjacent to the bone-cartilage interface in patients with RA. Expression of MMP-2 (gelatinase A), MMP-9 (gelatinase B), MMP-12 (macrophage metalloelastase), and MMP-14 (MT1-MMP) mRNA was detected in TRAP positive mononuclear and multinucleated cells by both RT-PCR and northern blot analysis. Immunocytochemistry for these MMPs showed that MMP-2 and MMP-9 were produced by both TRAP positive mononuclear and multinucleated cells, whereas MMP-12 and MMP-14 were produced by TRAP positive multinucleated cells. MMP-2 and MMP-9 were detected in the conditioned medium of TRAP positive mononuclear cells. TRAP positive mononuclear cells also induced the release of <sup>35</sup>S from prelabelled cartilage discs.

**Conclusion:** This study suggests that TRAP positive mononuclear and multinucleated cells located in the synovium at the cartilage-synovial interface produce MMP-2 and MMP-9, and may have an important role in articular cartilage destruction in patients with RA.

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Joint destruction in rheumatoid arthritis (RA) is characterised by invasion of the synovium into osteochondral tissue.<sup>1,2</sup> For bone absorption in RA, osteoclasts activated by inflammatory cytokines and chemokines are thought to have a central role, while certain cathepsins and matrix metalloproteinases (MMPs) secreted from osteoclasts seem to be key enzymes.<sup>3–7</sup>

In contrast with bone absorption in patients with RA, cartilage degradation at the cartilage-synovial interface seems to be more complex and involves several types of cells. The cartilage-synovial interface features particular cell types in RA, such as macrophage-like synoviocytes, fibroblast-like synoviocytes, osteoclasts, and chondrocytes.<sup>2</sup> Macrophage- and fibroblast-like synoviocytes that infiltrate into the cartilage seem likely to play a part in the degradation process, because such cells have the potential to produce many kinds of MMPs.<sup>8–11</sup> The chondrocytes in articular cartilage are also thought to be important in its destruction, because the stimulation of such cells by inflammatory cytokines (including tumour necrosis factor  $\alpha$  and interleukin 1) causes the secretion of many MMPs and initiates apoptosis.<sup>12,13</sup>

In the synovium at sites of cartilage destruction in patients with RA, tartrate resistant acid phosphatase (TRAP) positive mononuclear and multinucleated cells are often seen.<sup>14,15</sup> TRAP positive multinucleated cells, such as osteoclasts, can secrete various proteinases,<sup>16</sup> and this type of cell also seems to participate in cartilage destruction. In the growth plate, TRAP positive multinucleated cells are thought to be important in the process of replacing the hypertrophic chondrocyte zone with bone, and they are called chondroclasts.<sup>17</sup> However, the

**Table 1** Characteristics of the patients with RA at the time of surgery

Number of men/women	1/9
Age (years), mean (range)	61.1 (50–70)
CRP (mg/l), mean (range)	27 (9–46)
Disease duration (years), mean (range)	16.2 (1–28)
Number taking NSAIDs	5
Treatment during previous 6 months (number of patients)	
Gold salts	1
Bucillamine	1
Methotrexate	4
Prednisolone	5

CRP, C reactive protein; NSAIDs, non-steroidal anti-inflammatory drugs.

**Abbreviations:** DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte-macrophage colony stimulating factor; HE, haematoxylin and eosin; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction; SD, standard deviation; SDS, sodium dodecyl sulphate; SSC, saline sodium citrate; TPMoC, TRAP positive mononuclear cells; TPMuC, TRAP positive multinucleated cells; TRAP, tartrate resistant acid phosphatase; TBS, Tris buffered saline

**Table 2** Sequences of PCR primers for MMPs

	Primers (5'-3')		Expected product size bp
	Sense	Antisense	
MMP-1	GGGAACCTCGCTGGGAGCAAA	GGCCGAGTTCATGAGCCGCAAC	352
MMP-2	CAGCCTGGGACTGCCCTGAT	CAGGCCCTCCGGGTCTTCTC	400
MMP-3	CCTGGGACGCCAGCCAACTGTG	TGCTTGGGAAAGCCTGGCTCCAT	425
MMP-7	CCTCAGGAGGCGGGAGGCATGA	GCTCTCGCGCAAAGCCAATCA	425
MMP-8	GCCTCGCTGTGGAGTGCCTGACA	GCGAGCCCCAAAGAATGGCCAAA	414
MMP-9	GCCATCGCGGAGATTGGGAACC	CGGGGAACATCCGGTCCACCTC	400
MMP-10	ATGCGCAAGCCCAGGTGTGGAG	GCCCCAGGGAGTGGCCAAGTTC	418
MMP-11	CCCTGGCCCACTGTCACTCCA	GGCAGCATGGACCGGGAACCTC	393
MMP-12	TGAAAGTGACCGGGCAACTGGACA	TGTGCATCCCCTCCAATGCCAGA	370
MMP-13	GCGGGTTCTCTGATGTGGGTGA	GGCCGAACCTATGCGCAGCAAC	390
MMP-14	ATTCGAAGGCGTTCGCGTGT	CGGTCACTATCGGGCAGCACAA	398
MMP-15	CAATGAGCGCTCGGATGGAG	CGTTCACCGTCCGTGCCACCTC	263
MMP-16	GACAGTGCCCCACACCGCTCT	TAGGAGGCAAGCCCCGCCAGAA	260
MMP-17	GTGCGCTCTGGCAGCTGTACG	AGACGGGGCGCGGGTATCCTTC	403
G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCTGTTGCTGTA	450

PCR, polymerase chain reaction; MMP, matrix metalloproteinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

**Table 3** Amount of TRAP positive cells in the synovium and cartilage-bone interface

Patient	Sex	Age	Synovium	Cartilage-bone interface
1	F	60	418	96
2	F	63	77	410
3	F	57	583	410
4	M	70	1092	538
5	F	50	460	874
6	F	61	102	919
7	F	62	151	543
8	F	68	144	293
9	F	63	471	890
10	F	57	486	217
Average(SD)			398.4 (307.2)	519 (292.4)

TRAP, tartrate resistant acid phosphatase; SD, standard deviation; M, male; F, female.

role of TRAP positive cells in the destruction of articular cartilage has not been well investigated in patients with RA. We suggest that TRAP positive cells in the synovium and cartilage-synovial interface may be related to the destruction of articular cartilage in patients with RA.

To investigate the role of TRAP positive cells in the synovium and cartilage-synovial interface, we examined whether these cells produced MMPs in RA synovium. We also investigated whether mononuclear and multinucleated TRAP positive cells, generated in vitro from peripheral blood monocytes by incubation with RA synoviocytes, had the potential to produce various MMPs and to induce cartilage destruction.

## MATERIALS AND METHODS

### Tissue samples and preparation

Tissue samples, including the cartilage-synovial interface, were obtained during total knee arthroplasty from 10 patients with RA who gave informed consent. The American College of Rheumatology criteria were used for the diagnosis of RA.<sup>18</sup> Table 1 summarises the clinical backgrounds of these patients. After fixation in 4% paraformaldehyde at 4°C for 24 hours, tissue samples were decalcified in 20% EDTA for two hours in a microwave oven (H2800 Microwave Processor; Energy Beam Science Inc, Agawan, MA) at 50°C and then for 22 hours at 4°C.<sup>19</sup> Next, the samples were dehydrated through an ethanol series and embedded in paraffin. Sections (4 µm thick) were cut with a microtome and stained with haematoxylin and eosin (HE) stain, TRAP stain, and immunohistochemical stains.

### Quantification of TRAP positive cells in the synovium and cartilage-bone interface

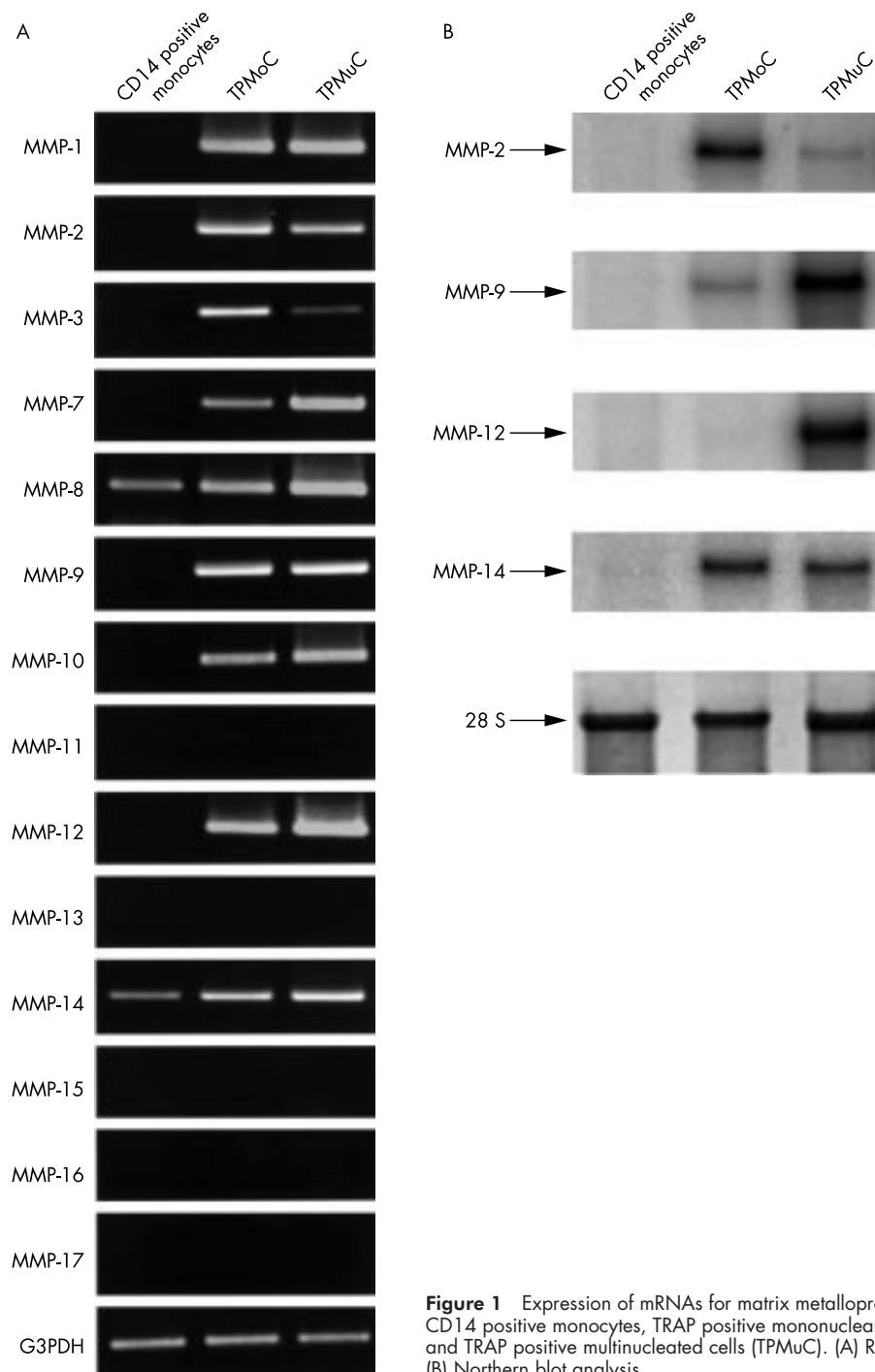
Tissue sections were deparaffinised and TRAP staining was performed using a commercial acid phosphatase leucocyte kit (Sigma, St Louis, MO). In the synovium and at the cartilage-bone interface, five areas (magnification ×100) were randomly observed with an HC2500 image analysis system (Fuji Photo Film, Tokyo, Japan) and the number of TRAP positive cells in each area was counted.

### Generation of mononuclear and multinucleated TRAP positive cells from peripheral blood monocytes by culture with RA synoviocytes

TRAP positive cells were generated as reported previously.<sup>20</sup> Briefly, CD14 positive monocytes were isolated from the peripheral blood of five healthy volunteers and cocultured for four weeks with RA synovial fibroblasts<sup>21</sup> in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT), 100 units/ml of penicillin (Gibco BRL), and 100 µg/ml of streptomycin (Gibco BRL) to produce TRAP positive mononuclear cells. After four weeks, the TRAP positive mononuclear cells became dominant. Over 97% of these cells were TRAP positive and their purity was confirmed cytochemically.<sup>20</sup> These cells differentiated into multinucleated cells after culture in DMEM in the presence of 1 ng/ml of granulocyte macrophage colony stimulating factor (GM-CSF; Genzyme, Cambridge, MA) for five days.

### Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from CD14 positive monocytes, TRAP positive mononuclear cells, and TRAP positive multinucleated cells using a TRIzol RNA isolation kit (Gibco BRL) according to the manufacturer's directions. After treatment with DNase I (Life Technologies, Rockville, MD), single stranded cDNA was synthesised using 3 µg of each RNA sample, 100 ng of random primers, and 200 U of SUPERScript II reverse transcriptase (Gibco BRL) in a total reaction volume of 20 µl. The cDNAs for various matrix metalloproteinases (MMP-1, 2, 3, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17) were amplified by PCR using the primer sets shown in table 2. Amplification was performed with 0.5 U of TaKaRa Ex Taq (Takara, Shiga, Japan) in a total reaction volume of 20 µl containing 1×reaction buffer, 200 µM of each dNTP, and 10 pmol of each primer. The PCR conditions were as follows: initial denaturation for two minutes at 94°C, 30 cycles of 30 seconds each at 94°C and 72°C (annealing at 72°C), and final extension for five minutes at 72°C. A fragment of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA



**Figure 1** Expression of mRNAs for matrix metalloproteinases by CD14 positive monocytes, TRAP positive mononuclear cells (TPMoC), and TRAP positive multinucleated cells (TPMuC). (A) RT-PCR analysis. (B) Northern blot analysis.

was also amplified as a control. Aliquots of the PCR products were subjected to electrophoresis on 1.5% agarose gels and visualised by ethidium bromide staining. In all experiments, reproducibility was confirmed in triplicate experiments.

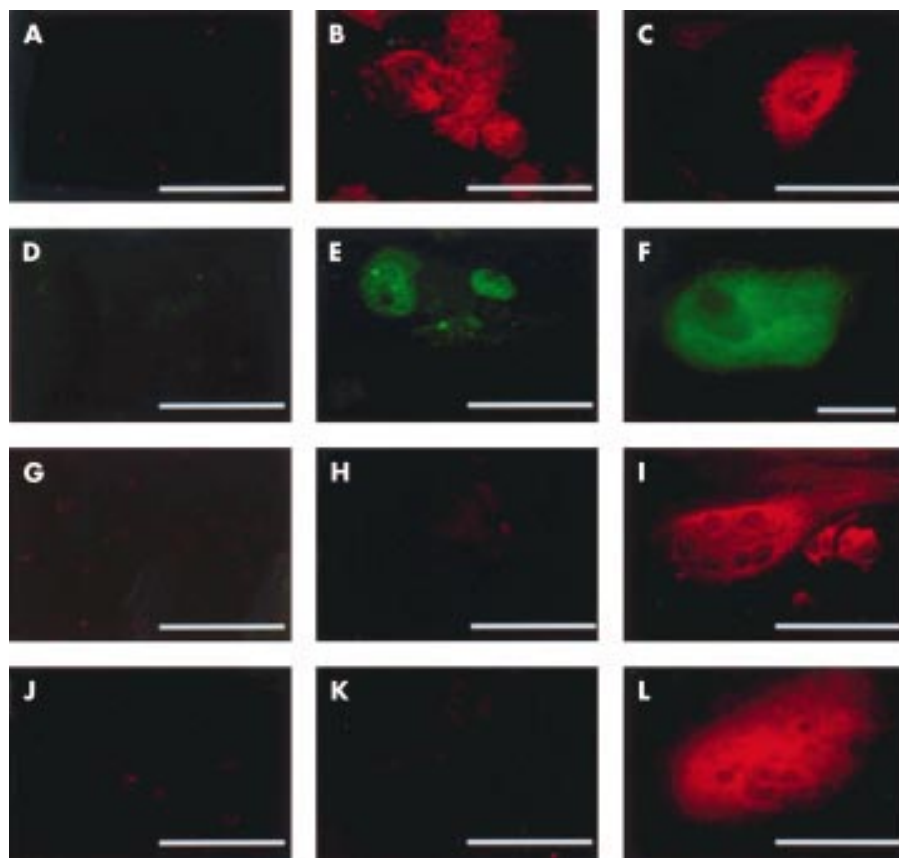
#### Northern blot analysis

Total RNA (2 µg) was subjected to electrophoresis on 1% agarose gel containing 2.2 M formaldehyde and then transferred to a nylon membrane by a standard method. Probes were prepared by <sup>32</sup>P labelling with Prime It (Stratagene, La Jolla, CA) using plasmids created with a PCR cloning kit (pGEM-T Easy; Promega, Madison, WI) from the corresponding genes described in table 2. Hybridisation was performed overnight at 65°C as described by Church and Gilbert.<sup>22</sup> After hybridisation, the membranes were sequentially washed twice at 45°C with

6×saline sodium citrate (SSC) plus 0.5% sodium dodecyl sulphate (SDS), twice with 2×SSC plus 0.1% SDS, and twice with 0.2×SSC plus 0.1% SDS. Hybridisation signals were detected with a BAS 2500 BioImage analyser (Fuji Photo Film).

#### Immunocytochemistry

TRAP positive mononuclear cells were suspended in DMEM (Gibco BRL) supplemented with 10% FCS (Hyclone), 100 U/ml of penicillin (Gibco BRL), 100 µg/ml streptomycin (Gibco BRL), and 1 ng/ml GM-CSF (Genzyme), and were seeded at  $2.5 \times 10^4$  cells/cm<sup>2</sup> on Lab-Tek chamber slides (Nunc, Inc, Naperville, IL). After incubation for five days, TRAP positive cells were fixed with 4% formaldehyde for 15 minutes and permeabilised using 0.2% Triton X100 for five minutes. Then the cells were sequentially incubated in phosphate buffered



**Figure 2** Expression of matrix metalloproteinases in CD14 positive monocytes, TRAP positive mononuclear cells (TPMoC), and TRAP positive multinucleated cells (TPMuC) as demonstrated by immunocytochemical analysis. MMP-2, MMP-9, MMP-12, and MMP-14 were expressed by TPMuC (C, F, I, and L), while additional expression of MMP-2 and MMP-9 was seen in TPMoC (B and E). (A, D, G, and J) CD14 positive monocytes; (B, E, H, and K) TPMoC; (C, F, I, and L) TPMuC. (A, B, and C) MMP-2; (D, E, and F) MMP-9; (G, H, and I) MMP-12; (J, K, and L) MMP-14. Bar = 100  $\mu$ m in A, D, G, and J; bar = 50  $\mu$ m in B, C, E, F, H, I, K, and L.

saline (PBS) plus 0.2% Triton X100 and 1% bovine serum albumin for 30 minutes, 2  $\mu$ g/ml of the primary antibody for the corresponding protein (MMP-2, MMP-14: Fuji Chem, Toyama, Japan; MMP-9: Santa Cruz, Santa Cruz, CA; MMP-12, Genzyme) in PBS (or isotype matched antibody as a negative control) at a similar concentration for one hour, and then 1  $\mu$ g/ml of the secondary antibody in PBS for one hour. As the secondary antibody, rhodamine conjugated rabbit antimouse immunoglobulins (Dako, Glostrup, Denmark) were used for MMP-2, MMP-12, or MMP-14, while FITC conjugated rabbit anti-goat IgG (Santa Cruz) was used for MMP-9. Finally, the slides were cover slipped with PBS-glycerol containing an anti-fading agent (*p*-phenylenediamine dihydrochloride; Sigma) and were observed under a fluorescent microscope (E800; Nikon, Tokyo, Japan) equipped with a standard mercury lamp.

#### Immunohistochemistry

Immunohistochemical staining was performed by the streptavidin biotin-peroxidase complex technique using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan) according to the manufacturer's instructions. Briefly, after blocking endogenous peroxidase and non-specific antigens, primary antibodies were applied to tissue sections and incubated for 24 hours at 4°C to detect MMP-2, MMP-9, MMP-12, and MMP-14. Isotype matched antibodies were used for the negative controls. After washing in PBS, the sections were incubated with the secondary antibody for 20 minutes at room temperature, followed by incubation with peroxidase conjugated streptavidin (Nichirei) for 20 minutes at room temperature and washing in PBS. Finally, colour was developed using 3,3'-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan), and the sections were counterstained with haematoxylin.

#### Immunohistochemical staining for MMPs combined with TRAP staining

Immunohistochemical staining for MMPs combined with TRAP staining was performed using the streptavidin biotin-alkaline phosphatase complex technique (Nichirei) according to the method recommended by the manufacturer. Briefly, after blocking non-specific antigenicity, primary antibodies were applied to the tissue sections and incubated for 24 hours at 4°C to detect MMP-2 and MMP-9. Isotype matched antibodies were used as the negative controls. After washing in Tris-buffered saline (TBS), the sections were incubated with the secondary antibody for 20 minutes at room temperature, followed by incubation with alkaline phosphatase conjugated streptavidin (Nichirei) for 10 minutes at room temperature and washing in TBS. Finally, colour was developed using a fast blue substrate kit (Nichirei). Endogenous alkaline phosphatase activity was blocked using an alkaline phosphatase blocking reagent (levamisole; DAKO). After immunostaining, TRAP staining was performed with a commercial acid phosphatase leucocyte kit (Sigma).

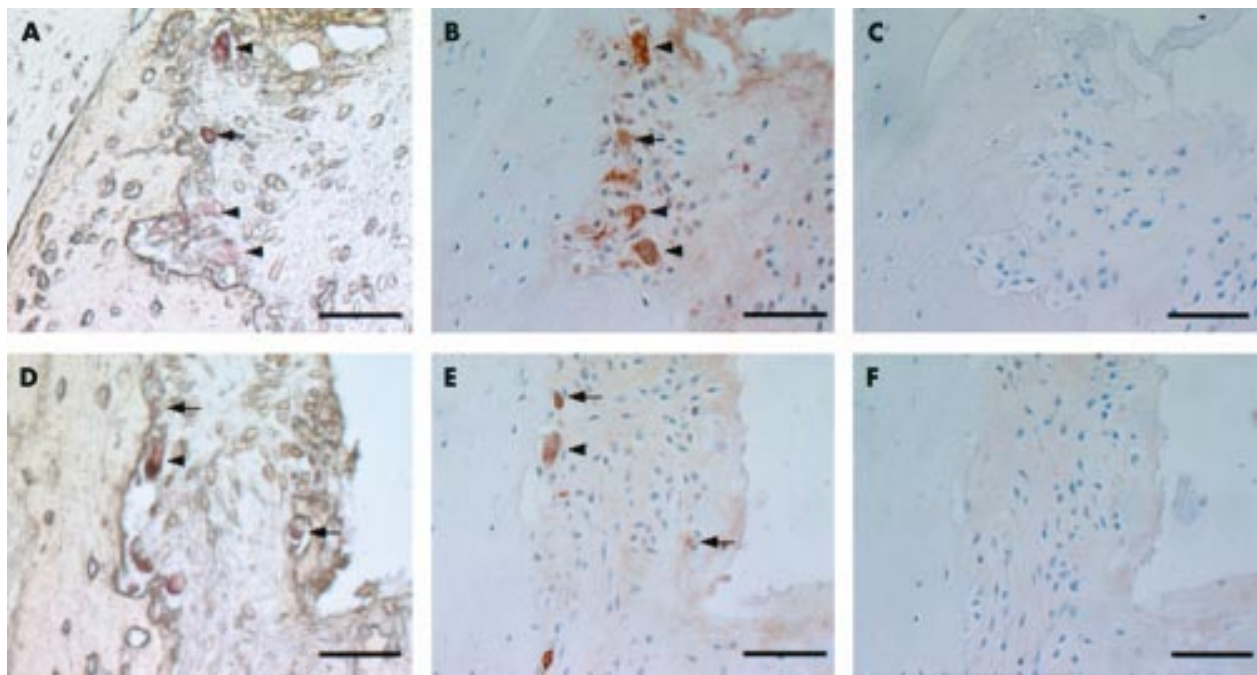
#### TRAP enzyme staining

TRAP enzyme was detected in paraffin sections using a commercial acid phosphatase leucocyte kit (Sigma).

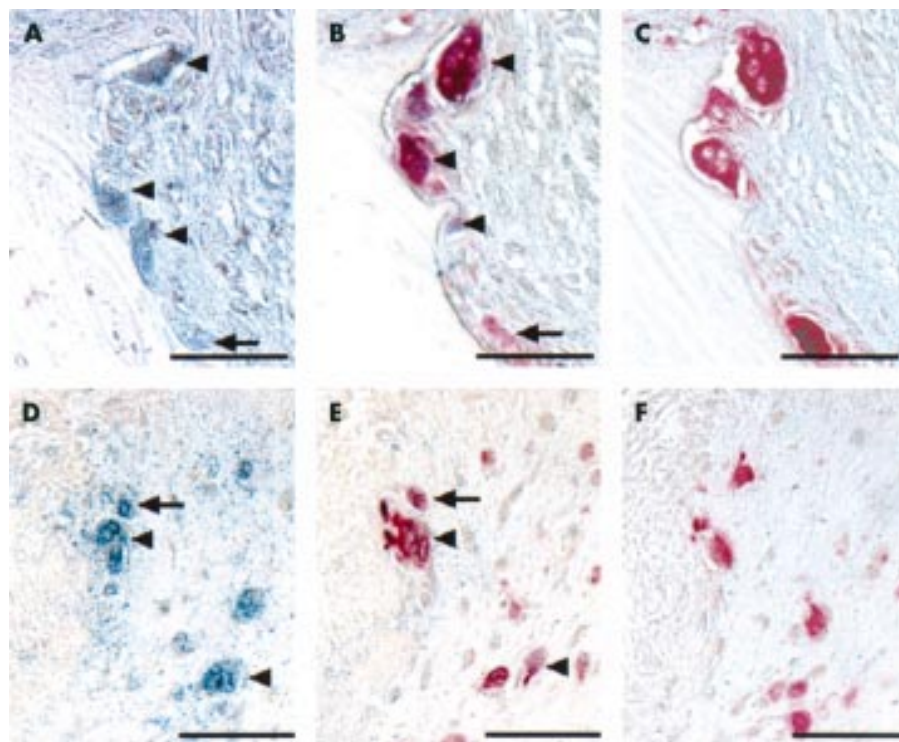
#### Quantification of MMP-2 and MMP-9

The concentrations of MMP-2 and MMP-9 in culture supernatants were measured using an enzyme linked immunosorbent assay (ELISA) kit (BIOTRAK; Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.





**Figure 3** Immunolocalisation of MMP-2 and MMP-9 in RA synovial tissue at the bone-cartilage interface. (A and D) TRAP staining, serial section. Mononuclear (arrows) and multinucleated cells (arrowheads) in RA synovial tissue at the bone-cartilage interface display TRAP activity. (B and E) Immunohistochemistry for MMP-2 and MMP-9. The TRAP positive mononuclear and multinucleated cells shown in A and D express MMP-2 and MMP-9. (C and F) Isotype controls for B and E. Haematoxylin counterstaining was performed in B, C, E, and F. Bar = 100  $\mu$ m.



**Figure 4** Immunostaining for MMP-2 or MMP-9 combined with TRAP staining of RA synovial tissue at the bone-cartilage interface. (A and D) Immunostaining for MMP-2 or MMP-9. Mononuclear cells (arrows) and multinucleated cells (arrowheads) in RA synovial tissue at the bone-cartilage interface express MMP-2 and MMP-9. (B and E) Immunostaining for MMP-2 or MMP-9 combined with TRAP staining. The TRAP positive cells are positive for MMP-2 and MMP-9. (C and F) Isotype controls for A and D combined with TRAP staining. Bar = 50  $\mu$ m.

#### Cartilage degradation assay

The cartilage degradation assay was originally described by Steinberg *et al.*<sup>23</sup> and was reported by Janusz *et al* and Scott.<sup>24,25</sup> Briefly, degradation of cartilage by TRAP positive mononuclear cells was assessed by culturing those cells in the

presence of a radiolabelled cartilage disc. Cartilage discs were prepared from bovine nasal septal tissues (obtained at the time of slaughter) using a 4 mm cork bore. Radiolabelling of the discs was done with <sup>35</sup>S labelled Na<sub>2</sub>SO<sub>4</sub> in the manner described by Janusz *et al* and the discs contained 5.0×10<sup>4</sup>–

$2.0 \times 10^5$  dpm of  $^{35}\text{S}$ . The discs were then subjected to five freeze-thaw cycles and heated at  $65^\circ\text{C}$  for 15 minutes to inactivate endogenous enzymes and cytokines, after which discs were stored at  $-20^\circ\text{C}$  before use.

Before performing the cartilage degradation assay, the discs were treated with DMEM (Gibco BRL) containing 0.4% collagenase I (Sigma) for 30 minutes at  $37^\circ\text{C}$  and were washed with DMEM containing 10% FCS. TRAP positive mononuclear cells ( $1.0 \times 10^5$ ) were added to each well of a 96 well culture plate and were cultured on the radiolabelled discs for seven days in 200  $\mu\text{l}$  of medium. For the neutralisation assay, 10  $\mu\text{M}$  and 100  $\mu\text{M}$  concentrations of an MMP-2/9 inhibitor (CALBIOCHEM; San Diego, CA) were added to the medium. Specific inhibition of both MMP-2 and MMP-9 at these concentrations by this inhibitor has been reported.<sup>26</sup> On day 7, 200  $\mu\text{l}$  of supernatant was removed from each well, added to 3 ml of ready-safe scintillation fluid (Beckman Instruments, Fullerton, CA), and counted in a scintillation counter (Quanta Smart for the Tricarb Liquid Scintillation Analyser; Packard Instrument Company, Meriden, CT). Then the residual isotope in each cartilage disc was measured in the same manner after the disc had been completely digested using 0.5 ml of tissue solubiliser (Beckman Instruments). The percentage of  $^{35}\text{S}$  release into the supernatant was calculated using the following formula:

$$100 \times (\text{dpm in supernatant}) / (\text{dpm in supernatant} + \text{disc})$$

All experiments were performed in triplicate.

## RESULTS

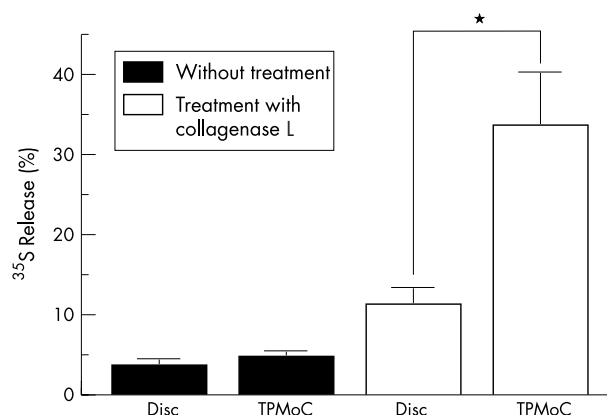
### Detection of TRAP positive cells in RA synovium near the cartilage-bone interface

Histological examination of the bone-cartilage interface was performed. The synovium adjacent to the bone-cartilage interface was found to contain TRAP positive cells. Table 3 gives details of the number of TRAP positive cells.

### TRAP positive mononuclear and multinucleated cells induced from peripheral blood monocytes by culture with RA synoviocytes express various MMPs

TRAP positive mononuclear and multinucleated cells were induced by coculture of peripheral blood monocytes with RA synoviocytes. We also performed coculture with RA synovial macrophages obtained from four patients with RA and RA synovial fibroblasts by the same method. However, most of the synovial macrophages disappeared within two weeks. Then MMP mRNA expression by the TRAP positive cells was assessed using RT-PCR and northern blot analysis. RT-PCR demonstrated that peripheral blood monocytes contained mRNA for MMP-8 and MMP-14 (fig 1A). In TRAP positive mononuclear and multinucleated cells, additional expression of the mRNAs for MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, and MMP-12 was detected. Northern blot analysis showed that the expression of MMP-2, MMP-9, MMP-12, and MMP-14 was increased in TRAP positive mononuclear and multinucleated cells (fig 1B). Although MMP-2 expression was significantly increased in TRAP positive mononuclear cells, MMP-9 and MMP-12 expression was higher in TRAP positive multinucleated cells. Expression of MMP-14 was similar in the two cell phenotypes. The amount of mRNA applied to each lane did not differ significantly, as determined by monitoring 28S ribosomal RNA. Other MMPs were not detectable by northern blot analysis (data not shown).

Immunocytochemical analysis of MMP-2, MMP-9, MMP-12, and MMP-14 was performed to confirm the expression of MMP proteins using monoclonal or polyclonal antibodies specific for these MMPs (fig 2). MMP-2 and MMP-9 were demonstrated in TRAP positive mononuclear cells, while additional expression of MMP-12 and MMP-14 was seen in TRAP positive multinucleated cells. These results were similar to those obtained by northern blot analysis. The other MMPs were not detected in monocytes or TRAP positive cells.



**Figure 5** Degradation of cartilage by TRAP positive mononuclear cells (TPMoC). Bars show the mean percentage and SD of  $^{35}\text{S}$  release on day 7 in 10 replicate cultures when radiolabelled cartilage discs were cultured in the presence (TPMoC) or absence of TPMoC (disc). The percentage degradation with and without collagenase I pretreatment is shown. \* $p < 0.01$  for disc v TPMoC (Mann-Whitney test).

### TRAP positive mononuclear and multinucleated cells in RA synovial tissue at the bone-cartilage interface express MMP-2 and MMP-9

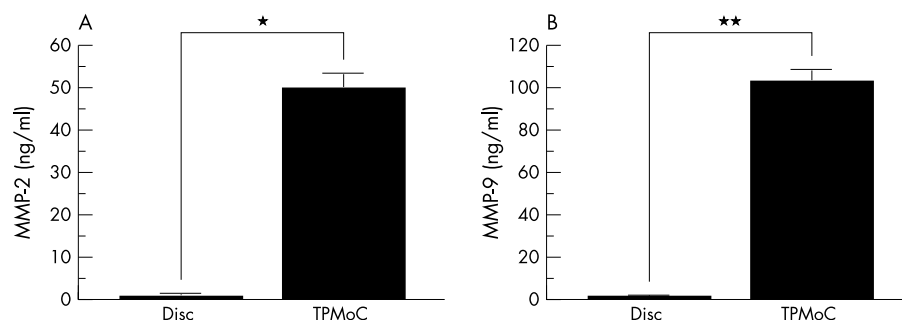
We examined MMP-2, MMP-9, MMP-12, and MMP-14 expression in the synovial tissue of 10 patients with RA by immunohistochemistry (fig 3). To detect the co-localisation of TRAP positive cells and MMP, we performed immunohistochemical phosphatase staining for a single MMP combined with TRAP staining in the same tissue section (fig 4). TRAP positive mononuclear and multinucleated cells in RA synovial tissue at the bone-cartilage interface showed strong expression of MMP-2 and MMP-9. Some of the synoviocytes at the bone-cartilage interface were also stained, but were only weakly positive. We did not detect MMP-12 or MMP-14 expression in RA synovial tissue (data not shown).

### TRAP positive mononuclear cells have the potential to induce the degradation of cartilage matrix by MMP-2 and MMP-9

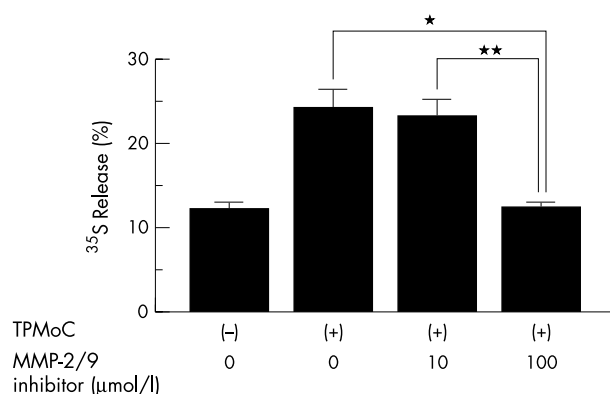
We examined whether TRAP positive mononuclear cells induced from monocytes by culture with RA synoviocytes had the potential to destroy cartilage. Release of  $^{35}\text{S}$  from labelled cartilage discs was increased in the supernatants of cultures containing TRAP positive mononuclear cells when compared with supernatants from cultures without these cells (fig 5). However, TRAP positive mononuclear cells did not induce degradation of cartilage discs without collagenase pretreatment. In the culture supernatants of TRAP positive mononuclear cells, high concentrations of MMP-2 and MMP-9 were detected (fig 6). When neutralisation experiments were performed using an MMP-2 and MMP-9 inhibitor to assess whether MMP-2 or MMP-9 released by TRAP positive mononuclear cells was responsible for induced cartilage degradation, it was found that degradation was completely inhibited by 100  $\mu\text{mol/l}$  of the inhibitor (fig 7). These results suggest that TRAP positive cells induced from peripheral blood monocytes by culture with RA synoviocytes have the potential to induce cartilage degradation by MMP-2 and MMP-9.

## DISCUSSION

In this study we clearly demonstrated that TRAP positive mononuclear and multinucleated cells exist in RA synovial tissue at the bone-cartilage interface and that these cells expressed MMP-2 (gelatinase A) and MMP-9 (gelatinase B). We also showed that TRAP positive mononuclear and multinucleated cells induced from peripheral blood monocytes by culture with RA synoviocytes expressed various



**Figure 6** Secretion of MMP-2 (A) and MMP-9 (B) into culture supernatant on day 7 in 10 replicate cultures when radiolabelled cartilage discs were incubated in the presence of TRAP positive mononuclear cells (TPMoC) or in the absence of such cells (disc). \*, \*\*p<0.01 for disc v TPMoC (Mann-Whitney test).



**Figure 7** A neutralisation experiment in the cartilage degradation assay was performed by adding a blocking agent for MMP-2 and MMP-9 to cultures of TRAP positive mononuclear cells. The inhibitor (100 μmol/l) blocked cartilage degradation. The mean percentage and SD shown in each column were calculated from 10 values of two identical experiments. \*, \*\*p<0.01 for 0 μmol/l v 100 μmol/l and 10 μmol/l v 100 μmol/l (Mann-Whitney test).

MMPs, especially MMP-2 and MMP-9, and that these generated mononuclear cells could cause cartilage degradation by producing MMP-2 and MMP-9. As far as we know, this is the first demonstration of cartilage degradation by TRAP positive cells from patients with RA.

We were aware of the aggregation of TRAP positive mononuclear and multinucleated cells in RA synovial tissues adjacent to areas of cartilage destruction, based on histological examination, and proposed that these cells might play some part in the destruction of articular cartilage.

First, we examined the expression of mRNAs for various MMPs by TRAP positive mononuclear and multinucleated cells induced from peripheral blood monocytes by coculture with RA synoviocytes. We found that TRAP positive mononuclear and multinucleated cells expressed mRNAs for a number of MMPs by RT-PCR, including MMP-1 (fibroblast-type collagenase), MMP-2, MMP-3 (stromelysin-1), MMP-7 (matrilysin), MMP-8 (neutrophil-type collagenase), MMP-9, MMP-10 (stromelysin-2), MMP-12 (macrophage metalloelastase), and MMP-14 (MT1-MMP). Northern blot analysis also detected some MMPs, including MMP-2, MMP-9, MMP-12, and MMP-14. Investigation of MMP protein expression by immunocytochemistry showed that MMP-2 and MMP-9 were expressed in both mononuclear and multinucleated cells, but MMP-12 and MMP-14 were only expressed in multinucleated cells. Discrepancies of mRNA and protein expression among the results of RT-PCR, northern blot analysis, and immunocytochemistry were observed. Variations in the sensitivity of the three methods and post-transcriptional regulation might have caused these discrepancies. However, clearly, TRAP positive cells generated in vitro had the potential to produce many

kinds of MMPs. We also examined MMP expression in RA synovial tissue adjacent to sites of cartilage destruction. Only MMP-2 and MMP-9 were detected in TRAP positive cells in vivo. Further investigation of MMP expression by TRAP positive cells is necessary, but we have shown that MMP-2 and MMP-9 may play a part in the tissue destruction that occurs in RA.

MMP-9 has been reported to be localised in monocytes/macrophages and osteoclasts.<sup>27,28</sup> In this study MMP-9 mRNA and protein were detected in TRAP positive mononuclear and multinucleated cells both in vitro and in vivo. These findings were consistent with a previous report.<sup>6</sup> Production of MMP-2 by osteoclasts is controversial. Previously, osteoclasts were reported to produce MMP-2,<sup>16</sup> but Ovejero *et al* recently reported that osteoclasts from rabbits did not express MMP-2.<sup>29</sup> It was also shown that MMP-2 can bind to  $\alpha_v\beta_3$  integrin and that MMP-2 produced by osteoblasts or other cells only binds to  $\alpha_v\beta_3$  integrin expressed by osteoclasts.<sup>30</sup> However, the present study showed that TRAP positive mononuclear and multinucleated cells expressed mRNA for MMP-2, these cells were stained positive by immunocytochemistry for MMP-2, and ELISA showed that mononuclear cells secreted MMP-2 into culture supernatants. Interestingly, MMP-2 expression was significantly up regulated in TRAP positive mononuclear cells in comparison with multinucleated cells. Further investigation is still necessary, but we believe that TRAP positive mononuclear cells and multinucleated cells can produce MMP-2.

MMP-2 and MMP-9 are gelatinases and seem to play a part in soft tissue destruction, but collagenases and stromelysin are also necessary for complete cartilage destruction. In the cartilage degradation assay, pretreatment with collagenase was necessary to allow cartilage degradation by TRAP positive mononuclear cells. Cooperation with synoviocytes and chondrocytes may be important in the mechanism of in vivo tissue degradation.

We previously reported that osteoclastogenesis was enhanced in the iliac bone marrow of patients with RA and that this was a possible cause of generalised osteoporosis.<sup>31</sup> Because TRAP positive mononuclear and multinucleated cells were identified in RA synovium,<sup>15</sup> we speculated that synovial fibroblasts participated in TRAP positive cell recruitment and in degradation of the osteochondral matrix. Therefore, we established a system to generate osteoclast-like cells from peripheral blood monocytes by culture with RA synoviocytes.<sup>20</sup> Recently, others have reported osteoclastogenesis when RA synovial macrophages and healthy peripheral blood mononuclear cells are cocultured with RA synovial fibroblasts.<sup>32-34</sup>

Our study showed some differences between TRAP positive cells in RA synovial tissue and TRAP positive cells generated in vitro. When generated in vitro, the TRAP positive mononuclear cells mainly expressed MMP-2 and MMP-9, and showed the same pattern of expression as TRAP positive mononuclear



cells detected in synovial tissue. In contrast, the in vitro generated TRAP positive multinucleated cells expressed MMP-2, MMP-9, MMP-12, and MMP-14, while the TRAP positive multinucleated cells in RA synovial tissue were only positive for MMP-2 and MMP-9. The sensitivity of immunocytochemistry might be different from that of immunohistochemistry, but the characteristics of in vitro and in vivo TRAP positive cells should be investigated further.

RA synovial fluid contains high levels of MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9, which have been implicated in cartilage destruction.<sup>35</sup> The major source of these proteins may be the synovium, where many kinds of MMP mRNAs have been detected.<sup>10</sup> However, considering that joint destruction progresses in a focal fashion, the proteolytic activity of RA synovial fluid, which bathes the entire joint, is not sufficient to explain the pathology of this disease. At the cartilage-synovial interface, where focal cartilage and bone destruction occurs, enhanced osteoclastogenesis has been observed.<sup>15</sup> In this study we also demonstrated focal aggregation of TRAP positive mononuclear and multinucleated cells at sites of destruction and positive staining of these cells for MMP-2 and MMP-9. Interestingly, TRAP positive mononuclear cells induced cartilage destruction in vitro by expressing MMP-2 and MMP-9.

MMP-9 expression by TRAP positive mononuclear cells suggested their phenotypic similarity with chondroclasts, which have been identified at the growth plate bone-cartilage interface.<sup>17</sup> We have already shown that in vitro generated TRAP positive multinucleated cells possess the characteristics of osteoclasts.<sup>20</sup> Blocking of this chondroclast-like and osteoclast-like cell lineage, the generation of which is mediated by RA synoviocytes, may be a possible way to prevent joint destruction in RA.

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